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Control of cortical axon elongation by a GABA-driven Ca^{2+} /calmodulin-dependent protein kinase cascade

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Abstract

Ca^{2+} signaling plays important roles during both axonal and dendritic growth. Yet, whether and how Ca^{2+} rises may trigger and contribute to the development of long range cortical connections remains largely unknown. Here we demonstrate that two separate limbs of CaMK kinase (CaMKK) - CaMKI cascades, CaMKK-CaMKI α and CaMKK-CaMKI γ , critically coordinate axonal and dendritic morphogenesis of cortical neurons, respectively. The axon-specific morphological phenotype required a diffuse cytoplasmic localization and a strikingly α -isoform-specific kinase activity of CaMKI. Unexpectedly, treatment with muscimol, a GABA_A receptor agonist, selectively stimulated elongation of axons but not of dendrites, and the CaMKK-CaMKI α cascade critically mediated this axonogenic effect. Consistent with these findings, during early brain development, *in vivo* knockdown of CaMKI α significantly impaired the terminal axonal extension, and thereby perturbed the refinement of the interhemispheric callosal projections into the contralateral cortices. Our findings thus indicate a novel role for the GABA-driven CaMKK-CaMKI α cascade as a mechanism critical for accurate cortical axon pathfinding, an essential process which may contribute to fine-tuning the formation of interhemispheric connectivity during the perinatal development of the central nervous system.

Keywords

axonal growth; calcium; CaMK; cerebral cortex; GABA; Kinase

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Introduction

The formation of cortical neural circuits requires precisely controlled development of axons and dendrites. While the molecular mechanisms underlying axon guidance in the central nervous system (CNS) has been intensively studied (Tessier-Lavigne and Goodman, 1996; Dickson, 2002), the intracellular signaling and cytoskeletal remodeling mechanisms implicated in the precise extension and targeting of axonal arbors still remain largely unsolved.

Ca^{2+} plays a central role in the regulation of neuronal morphogenesis. It is believed that there is an elevated optimal range for the intracellular Ca^{2+} concentration that supports maximal neurite outgrowth in various types of neurons (Kater et al., 1988; Gomez and Zheng, 2006). Ca^{2+} /calmodulin-dependent protein kinases (CaMKs), a major Ca^{2+} -dependent kinase family, are good candidates as potential downstream effectors of calcium elevation in neurons (Soderling and Stull, 2001; Hudmon and Schulman, 2002). While the essential role of CaMKII subfamily members in neuronal plasticity has been shown, much less is known about the function of the CaMKI/IV subfamily which forms several distinct kinase cascades downstream of CaMKK α and/or CaMKK β (Soderling, 1999; Hook and Means, 2001; Hudmon and Schulman, 2002; Bito and Takemoto-Kimura, 2003). The CaMKI family includes 4 isoforms: α (Nairn and Greengard, 1987), β /Pnck (Yokokura et al., 1997), γ /CL3 (Takemoto-Kimura et al., 2003) and δ /CKLiK (Ishikawa et al., 2003). Recently, a few number of reports from several laboratories, including ours, have started to suggest, *in vitro*, that CaMKI activity may participate in the regulation of neuronal morphology such as growth cone motility (Wayman et al., 2004), neurite outgrowth (Schmitt et al., 2004; Uboha et al., 2007), activity-dependent growth of dendrites (Wayman et al., 2006; Takemoto-Kimura et al., 2007), and stabilization of spines (Saneyoshi et al., 2008). However, evidence based on materials from genetically engineered animals is still scarce, and *in vivo* validation of such findings is still much awaited. Furthermore, in spite of the heavy expression of all CaMKI isoforms in the developing forebrain, there is yet little information as to what kinds of endogenous activity or extracellular ligands may influence the activity of CaMKI, during a perinatal period when only spontaneous Ca^{2+} transients are generated, and when synaptic activity-driven Ca^{2+} -mobilization is still missing.

We previously reported that a dendritic raft-anchored CaMK, CaMKI γ /CL3, plays an essential role in dendritic growth downstream of BDNF (Takemoto-Kimura et al., 2007). However, the exact context in which other CaMKI isoforms might contribute to neuronal morphogenesis remained obscure.

Here we show genetic and pharmacogenetic evidence which demonstrate that two separate limbs of CaMKK-CaMKI cascades, CaMKK-CaMKI α and CaMKK-CaMKI γ , critically coordinate axonal and dendritic morphogenesis of immature cortical neurons, respectively. Furthermore, we found that activation of GABA $_A$ receptors promoted axonal growth via the CaMKK-CaMKI α pathway. During perinatal brain development, *in vivo* knockdown of CaMKI α significantly impaired the terminal elongation of callosal axon projections in the somatosensory cortex. Taken together, our data suggest that a GABA-driven CaMK cascade may play a critical role in activity-regulated refinement of cortical axon wiring.

Materials and Methods

Construction of expression plasmids and RNA interference vectors

For RNAi experiments, short hairpin RNA (shRNA) vectors, co-expressing mRFP1 as a morphological tracer, were constructed essentially as described (Takemoto-Kimura et al., 2007). To create pSUPER-shCaMKI α and pSUPER-shCaMKI α #2, two complementary 60-bp oligonucleotides carrying antisense and sense sequences for CATTGTAGCCCTGGATGAC

(19-bp, corresponding to nucleotides 231-249 of mouse CaMKI α) and GATCAAGCACCCCAACATT (19-bp, corresponding to nucleotides 216-234 of mouse CaMKI α), respectively, were subcloned into the pSuper+mRFP1 plasmid backbone. pSUPER-shNeg was generated similarly except that an artificial 19-mer sequence (ATCCGCGCGATAGTACGTA) was used as a target as described (Takemoto-Kimura et al., 2007). This sequence was based upon a commercially available negative control siRNA sequence (B-Bridge International), and we confirmed that it had no significant identity to any known mammalian gene based on a BLAST search. Silent mutations were introduced into the shRNA target sequence of EGFP-tagged wildtype and mutant CaMKI α cDNAs to generate shRNA-resistant constructs (pEGFP-CaMKI α_{res} and related constructs). Short-hairpin RNA interference vectors against CaMKI α , CaMKI γ /CL3, and CaMKIV (shCaMKI α , shCaMKI γ /CL3, and shCaMKIV) selectively suppressed expression of GFP-CaMKI α , GFP-CaMKI γ /CL3, GFP-CaMKIV, respectively (Suppl. Fig. 2A, B). An antibody against CaMKIV (BD Transduction Laboratories) also confirmed these results. The potency of the knockdown was estimated to be about 70-80%, based on the reduction of overexpressed GFP-tagged proteins in Western blot analyses (Suppl. Fig. 2B). In keeping with this, and consistent with a transfection efficiency of >50% in our electroporation, we also detected a target-specific decrease of 40-50% in the amount of endogenous mRNA using a Real-Time PCR System (LightCycler 1.5, Roche Diagnostics) (Suppl. Fig. 2C).

Rat CaMKI α cDNA (Takemoto-Kimura et al., 2003) was inserted into pEGFPC1 vector (BD Clontech) to generate pEGFP-CaMKI α . The expression vector for a constitutively active form, pEGFP-CaMKI α_{CA} (286IHQS to 286EDDD, F307A) was created from pEGFPCaMKI α by site-directed mutagenesis. Similarly, a point mutation was introduced to generate pEGFP-CaMKI α_{K49A} . pCAG-EGFP-CaMKI γ /CL3 was as described (Takemoto-Kimura et al., 2007). CaMKK β wildtype and V269F cDNA (Tokumitsu et al., 2003) (a kind gift from Dr. Hiroshi Tokumitsu, Kagawa University, Japan) was subcloned into pEGFPC3. Mouse CaMKI β and CaMKI δ cDNAs were obtained from the German RZPD gene collection and RIKEN Genomic Science Center, respectively, and inserted into pEGFPC1 vector to generate pEGFP-CaMKI β and pEGFP-CaMKI δ . All constructs were verified by sequencing.

Gene targeting, neuronal culture and pharmacology

All animal experiments in this study were carried out in accordance with regulations and guidelines for the care and use of the experimental animals of the University of Tokyo, and approved by the institutional review committee of University of Tokyo Graduate School of Medicine.

CaMKK- α -KO mice were described before (Blaeser et al., 2006). CaMKK- β -KO mice were produced similarly by deleting exon 2 (where the ATG starts) through exon 6 of the CaMKK- β gene. A detailed characterization of CaMKK- β -KO mice will be described elsewhere (Blaeser, Chatila et al., under preparation). CaMKK- α - and CaMKK- β -KO mice were crossed to produce CaMKK α/β -double knockout (DKO) mice. The targeting strategy of CaMKI γ /CL3-KO mice was as described before (Takemoto-Kimura et al., 2007).

Dissociated cortical neurons were prepared and cultured from embryonic-day-19 Sprague-Dawley rats, or embryonic-day-17 C57BL/6 mice (wildtype as well as mutant mice), essentially as described previously (Takemoto-Kimura et al., 2007). In brief, dissected cortices were incubated for 10 min with 10 mg/ml trypsin type XI (Sigma) plus 0.5 mg/ml DNase I type IV (Sigma) at room temperature and mechanically dissociated in Hanks solution (pH 7.4) (Sigma) with 0.5 mg/ml DNase I type IV and 12 mM MgSO $_4$. Cortical neurons were transfected immediately after dissociation by electroporation using a Nucleofector (Amaxa Biosystems), plated onto poly-L-Lysine-coated 12 mm coverslips, glass-bottom dishes (Mat-Tek) or 6-well dish (BD Biosciences), and maintained in minimum essential medium (Invitrogen) containing

5 g/L glucose, 0.2 g/L NaHCO₃, 0.1 g/L transferrin (Calbiochem), 2 mM GlutaMAX-I (Invitrogen), 25 µg/ml insulin (Sigma), B-27 supplement (Invitrogen), and 10% fetal bovine serum. Cultures were maintained in 5% CO₂ at 37°C.

For inhibition and stimulation experiments, KN-93 (Calbiochem), STO-609 (TOCRIS), mevastatin (Wako, Japan), muscimol (TOCRIS), or BDNF (generously provided by Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan, by courtesy of Dr. Chikao Nakayama) were added to the medium of cultured neurons expressing mRFP1 at 6 h after plating at the final concentration of 10 µM (KN-93), 2.6 µM (STO-609), 10 µM (mevastatin), 1 µM (muscimol) and 50 ng/ml (BDNF), respectively. Bath application was performed by dissolving the reagents in one-half volume of the conditioned culture medium, and by mixing this gently with the remaining half of the original medium in the dish. No medium change was done onwards till fixation.

Immunocytochemistry, morphometric analyses, and visualization of raft-targeted proteins

For morphometric analysis, cortical neurons were transfected immediately after dissociation by electroporation using Nucleofector and plated onto 12 mm poly-L-Lysine-coated coverslips at the density of 5×10^5 cells (rats) or 7.5×10^5 cells (mice) per coverslip in 24-well plates. Dissociated cultures of rat and mouse cortical neurons and all measurements (axonal and dendritic length, axonal tip numbers) were performed at 2 days *in vitro* essentially as described (Takemoto-Kimura et al., 2007). Images of neuronal morphologies were captured based on immunoreactivities against GFP, mRFP1 or mCherry, using the Olympus BX51 microscopy system with a 20 x objective. Dendrites and axons were identified by standard morphological criteria as described (Takemoto-Kimura et al., 2007), and only neurons which possessed one clearly classifiable axon and one or more dendrites, were analyzed. All quantitative analyses were performed by an observer blinded to the identity of the transfected constructs, genotypes of transgenic mice, or treated drugs.

Immunostaining was carried out as described (Bito et al., 1996; Nonaka et al., 2006; Takemoto-Kimura et al., 2007). A rabbit anti-DsRed antibody (Takara, Japan) was used for quantitative morphometric analyses of RNAi, rescue and forced expression experiments, and a rat anti-GFP antibody (Nacalai Tesque, Japan) was used to detect coexpressed constructs. An anti-GM130 antibody (BD Transduction Laboratories) was used as a Golgi marker. As secondary antibodies, Alexa 488-, Alexa 594- conjugated anti-mouse, anti-rabbit and anti-rat IgG antibodies (Molecular Probes) were employed. Fluorescent images were taken by a confocal laser microscopy system (LSM 510META-V3.2, Carl Zeiss) built on an inverted microscope (Axiovert 200M, Carl Zeiss) with the 40x objective (Plan-Neofluar 40x/NA 1.3, oil, Carl Zeiss), or using a CCD camera-based imaging analysis system (an Olympus BX51 equipped with a DP-70 camera). Visualization of raft-targeted proteins was carried out as described (Takemoto-Kimura et al., 2007).

Western blot analysis

For Western blot analysis, cortical neurons were transfected with pSUPERshNega or pSUPER-shCaMKI α by electroporation using a Nucleofector and plated at a density of 5×10^6 cells in a 6-well dish. At 2 DIV, the cells were lysed in lysis buffer containing 50 mM Tris-HCl (pH 6.8), 2% SDS, and 10% glycerol. A rabbit anti-CaMKI α antibody (Uezu et al., 2002) was used (a kind gift from Drs. Kohji Fukunaga and Jiro Kasahara, Tohoku University, Japan). Chemiluminescence detection was performed using horseradish peroxidase-conjugated anti-rabbit IgG and ECL-Plus reagent (Amersham Biosciences).

Calcium imaging

Fluorescent calcium imaging was performed essentially as described previously (Furuyashiki et al., 2002; Takemoto-Kimura et al., 2007). Twenty-four hours after plating, cortical neurons on glass-bottom dishes were loaded with Fluo-4/AM (2.5 μ M, Dojindo laboratories, Kumamoto, Japan) for 30 min at room temperature. After wash, cells were incubated at 37 °C in a stage CO₂ chamber (Tokai Hit Co., Ltd, Shizuoka, Japan) equipped on an LSM510META (Carl Zeiss). After baseline recording, a medium containing 20 \times muscimol (final concentration 1 μ M) was gently bath-applied. Fluorescence changes in the cell bodies of individual cells were analyzed using Metamorph or Image J software, and data are expressed as $\Delta F/F_0$.

In utero electroporation. data acquisition and quantification of the terminal arborization of callosal axons

In utero electroporation was performed as described previously (Mizuno et al. 2007). Equal amount of pSUPER-vectors (2 μ g/ μ l) and pCAG-EGFP (2 μ g/ μ l) were mixed together with a dye Fast Green (0.05%, Wako, Japan) for injection into the lateral ventricle. The postnatal brains (P16) were fixed by transcardial perfusion of 4 % PFA in 0.1 M phosphate buffer followed by overnight immersive fixation in 4% PFA in PBS and then transferred to 30% sucrose in PBS for 1-2 days at 4 °C. Serial coronal brain sections were prepared at 50 μ m thickness by a cryostat (HM560, Microm), and every one section out of 4 were immunostained. Sections were permeabilized in 0.3% Triton X- 100 in PBS and then blocked in 5% normal goat serum, 1% BSA, and 0.3% Triton X- 100 in PBS followed by fluorescent immunostaining of EGFP. Sections were counterstained with DAPI (Molecular Probes). Quantitative analyses were performed and compared using the utmost posterior section of the stained sets that included the corpus callosum.

Confocal images were taken (LSM 510META-V3.2, Carl Zeiss) with a 10x objective (Plan-Neofluar 10x/NA 0.3, air, Carl Zeiss) with 10 μ m optical sectioning. Z projection images taken at 512 \times 512 pixels were acquired by average projection mode and background was subtracted, and the intensity was normalized by maximal intensity in the white matter. For one dimensional fluorescence intensity profile analysis in Fig. 7C, a rectangular zone (nominal width set at 100 pixels) was drawn along the vertical axis from the pial surface to the white matter and the average pixel intensity projected onto the vertical axis was calculated. To quantify the impairment of the cortical wiring in Fig. 7D, average intensity in a rectangle region (100 pixels in width) in the cortex was divided by that of in the white matter. Three to 5 pups were used for quantification. All calculations were performed using Metamorph software (Ver. 7, Molecular Devices).

Statistical analyses

Statistical analyses were run separately for axonal and dendritic datasets throughout our study, while using scattered diagrams of paired data of axonal and dendritic lengths (“orthogonal plots”). Statistical analyses were performed using Prism 4.0 (GraphPad Software). Student’s t test was used for comparisons of two groups. One-or two-way analysis of variance (ANOVA) with post hoc Tukey-Kramer or Bonferroni test was used for factorial analysis between more than three groups. Kolmogorov-Smirnov test was applied to Fig. 1C, D. All data are shown as mean \pm standard error of mean (SEM), unless otherwise mentioned, and shaded regions in orthogonal plots graphically depict the zone of mean \pm 2*SEM on both axes to facilitate the evaluation of the phenotypes.

Results

CaMKK pathway regulates axonal and dendritic growth during early stages of cortical development

We previously reported that, a dendritic raft-anchored CaMKI γ /CL3 (Takemoto-Kimura et al., 2003) plays an essential role in dendritic growth downstream of BDNF during the morphological maturation of cortical neurons (Takemoto-Kimura et al., 2007). As 4 distinct CaMKI isoforms (α , β , γ /CL3 and δ) and CaMKIV are activated by upstream CaMK kinases (CaMKK)- α and β , we sought to test how potently neuritogenesis was disturbed in cultured cortical neurons generated from CaMKK α/β -double knockout (DKO) mice. To specifically identify the genotype contribution to either axonal or dendritic growth, we orthogonally plotted the dendritic length (i.e. total length of all dendritic processes) and axonal length (i.e. total length of all axonal processes including branches) for each GFP-expressing cortical neuron blindly chosen from multiple fields of view (Fig. 1A, B and Supplemental Fig. 1). While cortical neurons from CaMKI γ /CL3 knockout mice revealed a strikingly dendrite-specific deficit (Fig. 1A-D), we found that both axons and dendrites were significantly shortened in cortical neurons from DKO mice, as compared to neurons from wild-type (WT) mice (Fig. 1A-F). Exposure to KN-93, which blocks all CaMK species (CaMKII, CaMKI, CaMKIV, and CaMKK), also reduced both total axonal and dendritic lengths (Fig. 1G, H). Specific blockade of the CaMKKs, using STO-609, a selective inhibitor for CaMKKs (Tokumitsu et al. 2002), resulted in a quantitatively similar impairment (Fig. 1G, H). Together, these genetical and pharmacological experiments clearly demonstrated that CaMKK-mediated CaMK cascades played critical roles both in axonogenesis and dendritogenesis of immature cortical neurons, consistent with a prior work on other cell types (Wayman et al., 2004). Furthermore, our data pointed to the presence of a selective CaMKK-CaMK cascade which strongly supported cortical axonal growth in a manner that was distinct from the dendritic contribution of CaMKI γ .

Suppression of CaMKI α expression specifically impairs axonal but not dendritic growth

In order to identify which of CaMKI or CaMKIV isoform(s) was involved in regulation of the axonal growth, we designed several short hairpin-type pSUPER vectors which were targeted to specific isoforms of the CaMKI/IV subfamily members. In this RNAi experiment, we also coexpressed a PGK promoter-driven mRFP1 as a morphological tracer. In a control experiment, polarized cortical neurons grown for 48 h typically grew 5 ~ 6 dendrites and a single axon. Knockdown using an shCaMKI α vector was prominent enough such that even an overexpressed GFP-CaMKI α became barely detectable 48 h after transfection, while the control mRFP1 expression level remained unchanged (Fig. 2A). Strong suppression of endogenous CaMKI α expression in shCaMKI α -transfected neurons was also demonstrated by Western blot analysis using an anti-CaMKI α antibody (Fig. 2B). A lack of cross-knockdown effects across α -, γ - CaMKI isoforms and CaMKIV was verified (Supplemental Fig. 2).

Under these conditions, shCaMKI α -treated neurons showed unchanged dendritic growth, but had a markedly shorter axon (Fig. 2C, D). Under the same conditions, in contrast, CaMKI γ /CL3 knockdown specifically blocked dendritic, but not axonal, outgrowth (Fig. 2E and (Takemoto-Kimura et al., 2007)), while CaMKIV knockdown had no effect (Fig. 2E). The striking specificity in CaMKI α 's axonal phenotype was replicated even when axonal growth was measured under conditions in which shCaMKI α -transfected neurons were kept in suspension culture for an extended period (48 h) prior to plating, to ensure a maximized knockdown efficiency (Supplemental Fig. 3). The impairment in axonal growth observed in CaMKI α -diminished neurons was rescued by expression of an shCaMKI α -resistant WT-CaMKI α (WT_{res}), but not by that of an shCaMKI α -resistant kinase-inactive CaMKI α (K49A_{res}), demonstrating the requirement of the kinase activity of CaMKI α (Fig. 2F). Taken

together, our results strongly implicated the CaMKK-CaMKI α cascade as a critical player in the control of cortical axonal growth.

Two separate CaMKK-CaMKI cascades control cortical axonal and dendritic growth

In keeping with this robust selectivity in the knockdown experiments, forced expression of either one of the 4 CaMKI isoforms revealed that total axonal length was stimulated only by an increase in CaMKI α , while dendrite growth was promoted only by CaMKI γ /CL3 expression (Fig. 3A, B). No change in primary axon number was detected in CaMKI α -overexpressing neurons, suggesting that CaMKI α did not act on axon specification per se (data not shown). Most critically, expression of a constitutively active CaMKI α (CaMKI α CA), was sufficient to rescue the axonal deficit, but without altering dendritic atrophy, in cortical neurons from DKO mice (Fig. 3C) or in WT neurons treated with STO-609 (Fig. 3D). However, forced expression of CaMKI α WT, which enzymatically remains inactive in the absence of CaMKK activity, had no effect in either of these backgrounds (Fig. 3C, D). In a parallel experiment, both axonal and dendritic defects in WT neurons treated with STO-609 were rescued by transfection of a STO-609-resistant CaMKK β V269F mutant (Tokumitsu et al., 2003) (Fig. 3E).

Taken together, these data strongly implicated the CaMKK-CaMKI α and CaMKK-CaMKI γ cascades as parallel pathways acting independently in the promotion of axonal and dendrite growth, respectively, in cultured cortical neurons.

Both localization and kinase specificity of CaMKI α play important roles in CaMKI α -dependent axonal growth

Our data, so far, suggested that the axonogenic action of CaMKI α manifested in a manner that was completely orthogonal and independent to the dendritogenic effect mediated by CaMKI γ /CL3, in spite of a high degree of structural identity (71% amino acid identity in the catalytic domain sequences). What then discriminated the distinct function of these two kinases?

To identify the molecular determinants involved in axonogenic and dendritogenic selectivity of the CaMKK-CaMKI cascades, we generated CaMKI α / γ chimeras such that each kinase domain was paired with either cytosolic or Golgi / raft localization signals in the C-terminus (Fig. 4A and (Takemoto-Kimura et al., 2007)). We then tested their potencies to rescue the defect due to knockdown of endogenous CaMKI α . As expected, forced expression of an shCaMKI α -resistant WT-CaMKI α ($I\alpha_{res}$) rescued the axonal impairment in CaMKI α knockdown neurons (Fig. 4B). The WT-CaMKI γ /CL3 ($I\gamma$), however, promoted dendritic growth without showing any effect on axonal deficit. CaMKI α is believed to be freely diffusible. On the other hand, the C-terminal region of CaMKI γ /CL3 is lipidified by prenylation and palmitoylation, targeting it preferentially into lipid rafts which are highly abundant in dendrites and in Golgi (Takemoto-Kimura et al., 2007) (Fig. 4A and Supplemental Fig. 4). A dendritic raft-targeted mutant of CaMKI α , GFP-CaMKI α +C_{term} ($I\alpha_{raft-res}$), was unable to rescue the axonal impairment in CaMKI α knockdown neurons (Fig. 4B). A cytoplasmic, raft-excluded mutant of CaMKI γ /CL3, namely CaMKI γ /CL3 Δ C_{term} ($I\gamma_{cyto}$), had no ability, either (Fig. 4B), contrary to our expectations. Thus surprisingly, CaMKI-mediated selectivity of neurite growth might not be simply determined by the localization of a CaMKI α or CaMKI γ in or out of the membrane rafts.

To further confirm this, the chimeras were expressed in the background of CaMKI γ /CL3-knockdown neurons. Expression of an RNAi-resistant WT-CaMKI γ /CL3 ($I\gamma_{res}$) rescued the dendritic impairment, while WT-CaMKI α ($I\alpha$) promoted axonal growth without an effect on dendrite impairment (Fig. 4C). Again however, neither a freely diffusible CaMKI γ /CL3 Δ C_{term} ($I\gamma_{cyto-res}$), nor a raft-targeted CaMKI α , CaMKI α +C_{term} ($I\alpha_{raft}$), had any effect

(Fig. 4C). Furthermore, forced expression of CaMKI α , CaMKI γ , and CaMKI α/γ chimeras in a naïve background revealed that total axonal length was stimulated only by an increase in CaMKI α (Fig. 4D). Together, these data provide strong functional evidence in support of the notion that CaMKK-CaMKI α and CaMKK-CaMKI γ are not duplicative mechanisms with simply altered targeting of downstream kinases, but are genuinely segregated cascades that are divergent at the level of kinase substrate specificity.

GABA is one of the physiological ligand acting upstream of CaMKI α to promote axonal growth during early stages of cortical development

The biological significance of this specificity could be demonstrated if the physiological signal triggering the axonogenic effect of CaMKI α was identified. To this end, we searched for a potential extracellular ligand which induced intracellular calcium elevation and potentially stimulated axonal growth. We found that muscimol, a GABA_A receptor agonist with a known excitatory action during perinatal development (Owens et al., 1996; Represa and Ben-Ari, 2005), specifically promoted elongation of axons, but not of dendrites, in cultured cortical neurons (Fig. 5A, B). Under the same conditions, we confirmed that BDNF had a complementary growth effect largely selective for dendrites (Takemoto-Kimura et al., 2007).

To test the extent of requirement for GABA, we added bicuculline, a GABA_A receptor antagonist, in the medium and found that axonal growth was rather selectively impaired (Fig. 6A). Furthermore, we confirmed that muscimol application triggered a strong Ca²⁺ influx in our cortical neurons (Fig. 6B, C). In keeping with this, forced expression of KCC2, a neuronal K⁺/Cl⁻ co-transporter that lowers intracellular Cl⁻ concentration, and which is up-regulated during development to convert the GABA action from excitation to inhibition (Rivera et al., 1999), impaired both constitutive and muscimol-stimulated axonal growth (Supplemental Fig. 5). Pharmacological blockade of all CaM kinases using KN-93 (Supplemental Fig. 6), or of CaMKK using STO-609 (Fig. 6D), completely blocked the axonogenic muscimol effect. CaMKI α RNAi (shI α), but not CaMKI γ /CL3 RNAi (shI γ), selectively impaired muscimol-stimulated axonal growth (Fig. 6E), and this effect was rescued by co-expressing an shCaMKI α -resistant CaMKI α WT (I α WT_{res}), but not CaMKI γ /CL3 WT (I γ WT) (Fig. 6F). Thus a CaMKK-CaMKI α cascade may critically mediate GABA_A-stimulated axon outgrowth during the early development of a cortical neuron.

Contribution of CaMKI α in fine-tuning axonal pathfinding in vivo

We finally tested the *in vivo* relevance of these findings by investigating the function of CaMKI α during activity-dependent cortical wiring *in vivo*. The callosal axons that originate from layer II/III pyramidal neurons of the somatosensory cortex are known to elongate and target themselves to the border between the S1 and S2 areas of the contralateral cortex, where they suddenly turn and grow into the cortical layers and develop their terminal branches mainly at layers II-III and V. Previous reports demonstrated that reduction of neuronal excitability by overexpression of an inwardly rectifying potassium channel, Kir2.1, impaired such layer-specific development of the terminal branches in the visual cortex (Mizuno et al., 2007) and in the somatosensory cortex (Wang et al., 2007). Furthermore, premature elimination of excitatory GABA drive by forced expression of KCC2 or knockdown of NKCC1 in newly born cortical neurons dramatically perturbed the morphological maturation of the dendrites (Cancedda et al., 2007; Wang and Kriegstein, 2008), or of the terminal callosal axon branches (H.M., T. H., Y.T., unpublished data).

If the morphogenetic effect of excitatory GABA required Ca²⁺ signalling, could the CaMKK-CaMKI α pathway perhaps mediate activity-dependent control of callosal axonal extension? To test this, CaMKI α was knockdown in the somatosensory layer II/III neurons by in utero electroporation at E15.5, and an effect on axonal growth was examined. At P16, control neurons

terminated their axons into a restricted region (border of S1/S2 area) in the contralateral cortex and extensively developed their terminal branches into layers II/III and V (Fig. 7A). CaMKI α -knockdown neurons extended interhemispheric axonal projections in the white matter, suggesting the CaMKI α may not be absolutely required for midline crossing and progression of axon fibers (Fig. 7B). However, their terminal axonal extension into the cortical layers was severely diminished, especially in layers II/III (Fig. 7C, D). These results indicate a developmentally critical role of CaMKI α in activity-dependent regulation of cortical connectivity *in vivo*.

Discussion

Differential control of cortical axonogenesis and dendritogenesis by activation of CaMKI α and CaMKI γ /CL3

In a previous work (Takemoto-Kimura et al., 2007), we showed that a lipid-modified CaM kinase CaMKI γ /CL3 (a membrane-anchored CaMKI isoform) was directed to the dendrites upon raft insertion and could potentially promote early dendritic development, with little effect on axon outgrowth, in cultured cortical neurons. In striking contrast to CaMKI γ /CL3, we here demonstrate that a cytosolic sister kinase, CaMKI α , has a complementary role: it has little role in dendritogenesis, but is necessary and sufficient to promote axonogenesis in the same preparation. Additionally, our present work established that CaMKI α regulates axonal extension *in vivo*. Further rigorous quantitative studies are awaited to establish the potential role of other CaMKK-CaMK signaling pathways in cortical neuritogenesis in general.

How can such specificity of axonal / dendritic growth be regulated by two separate yet structurally resembling kinases lying downstream of the same CaMKKs? The chimeric kinase experiments (Fig. 4) strongly suggested that the diverging kinase substrate specificities and the dissimilarity in subcellular localization (cytosol vs dendritic rafts) might provide a basis for the strikingly differential effect of CaMKI α and CaMKI γ /CL3 during axonal and dendritic development. In support of this functional segregation between the two distinct CaMKK-CaMKI cascades, we identified an extracellular ligand, GABA, which specifically stimulated axonal growth via CaMKI α (this study), while BDNF selectively promoted dendritic growth via CaMKI γ (Takemoto-Kimura et al., 2007), during an early developmental stage of cortical neurons.

In principle, BDNF could rather selectively act on dendrites in part because of the strong affinity of the active TrkB receptor to lipid rafts (Suzuki et al., 2004), which are enriched on dendrites. At this point, however, how GABA stimuli could possibly generate an axon-specific effect remains rather unclear, although preliminary Ca²⁺ imaging experiments indicated that GABA_A stimulation might trigger growth cone-localized Ca²⁺ transients (S.K., H.F., S.T-K. and H.B., unpublished data). It is noteworthy that many potential *in vitro* substrates of CaMKI α have previously been associated with axonal or presynaptic functions. These include synapsin I (Nairn and Greengard, 1987), myosin II regulatory light chain (MRLC) (Suizu et al., 2002), Numb and Numbl (Tokumitsu et al., 2005), microtubule affinity regulating kinase 2 (MARK2/Par-1b) (Uboha et al., 2007), and β Pak-interacting exchange factor (β PIX) (Saneyoshi et al., 2008). While some of these known substrates of CaMKI may potentially underlie a part of early axonal growth, further work is clearly needed to fully elucidate how an axonogenic substrate may be activated via phosphorylation by CaMKI α .

A pivotal role for a GABA-driven CaMKK-CaMKI α cascade in controlling axonal morphogenesis during early development

In this work, we identified a crucial role for GABA in controlling cortical axon outgrowth during early development via a CaMKK-CaMKI α cascade. In immature cortical neurons, what

is the mechanism by which GABA can stimulate axonal development in a CaMKI-dependent manner? Recent studies showed that GABA_A receptors activation has potent excitatory effects in immature, but not in mature, neurons (Ben-Ari et al., 2007). The excitatory action of GABA was demonstrated to be caused by a high basal Cl⁻ concentration in immature neurons, due to a high amount of the Na⁺-K⁺-2Cl⁻ cotransporter (NKCC1) which favors Cl⁻ influx, while the K⁺-Cl⁻ cotransporter (KCC2) largely responsible for Cl⁻ efflux is still low in expression (Payne et al., 2003). Because of elevated intracellular Cl⁻ concentration in immature neurons, GABA_A receptors activation thus induces depolarization (Ben-Ari et al., 2007), thereby likely triggering the opening of voltage-gated Ca²⁺ channels, which then generates enough Ca²⁺ influx leading to CaMKK-CaMKI α activation.

During early development, it is now known that GABA controls a variety of biological processes. Our work has only addressed the significance of the CaMKK-CaMKI α cascade in GABA-mediated cortical axonogenesis during the perinatal period. Whether other GABA-regulated processes may also be mediated by CaMKI α clearly remains to be investigated. For instance, the process of cortical migration has also been reported to be regulated by GABA through signal transduction pathways involving Ca²⁺, both *in vitro* (Behar et al., 1996; Behar et al., 1998; Behar et al., 2000) and *in vivo* (Heck et al., 2007). Interestingly, treatment with calmidazolium, an inhibitor of calmodulin, reduces the migration rate in cerebellar granule cells (Kumada and Komuro, 2004). Further studies are needed to determine whether the CaMKK-CaMKI α cascade may play additional roles in such developmental processes as well.

A CaMKK-CaMKI α pathway may regulate fine-sculpting of cortical wiring

We here established the critical importance of an axonogenic GABA-CaMKK-CaMKI α pathway during early development *in vitro*. Moreover, this study indicated that CaMKI α regulated activity-dependent extension of terminal cortical axons *in vivo*. Interestingly, premature elimination of excitatory GABA action by forced expression of KCC2 in newly born cortical neurons dramatically perturbed the morphological maturation of the dendrites (Cancedda et al., 2007), or of the terminal callosal axon branches (H.M., T.H., and Y.T., unpublished data). Our present findings thus uncover an unexpected role of the CaMKK-CaMKI α cascade as one key mechanism in GABA-driven activity-dependent regulation of cortical connectivity. More studies are needed to establish whether and how other Ca²⁺-mobilizing signals (e.g. BDNF) may spatially and temporally interact and perhaps cooperate with such an axonogenic GABA-CaMKK-CaMKI α pathway. Finally, our data lend support to the existence of a perinatal time window of structural refinement, during which spontaneous Ca²⁺ signalling regulated by trophic factors, guidance signals and ambient neurotransmitters, such as BDNF or GABA, critically fine-tunes cortical connectivity, perhaps even prior to the receipt of the earliest sensory cues.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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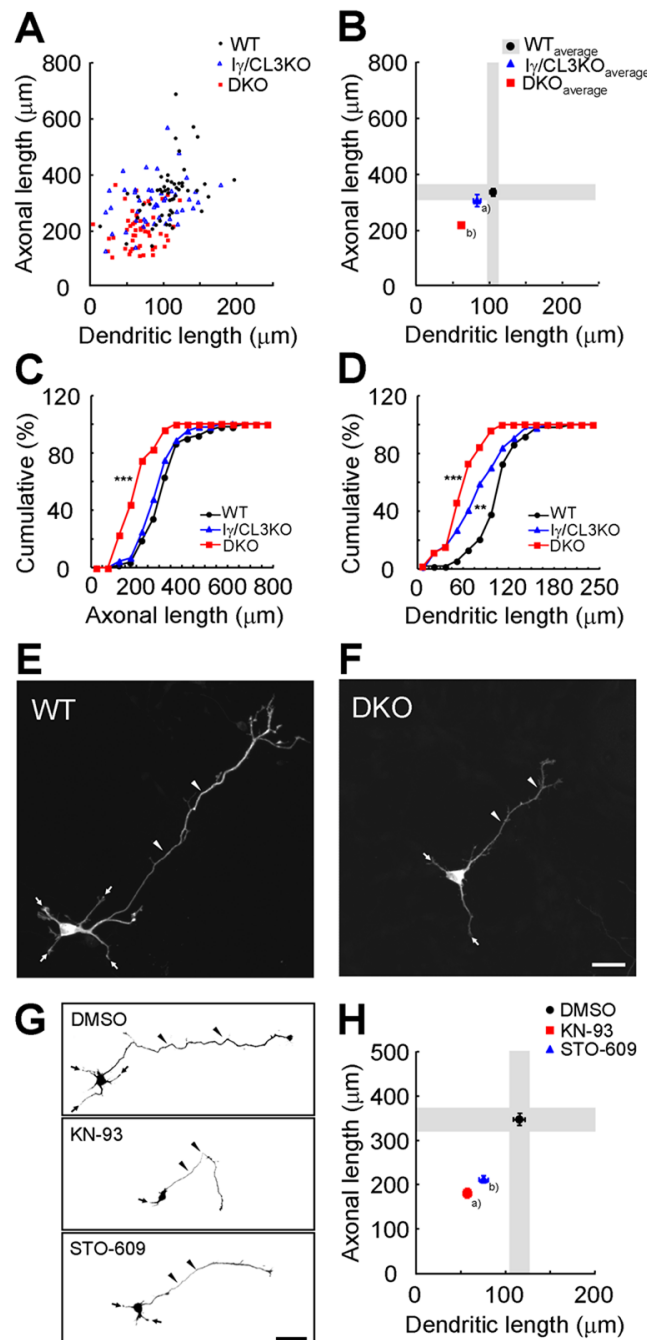


Figure 1. CaMKK-dependent CaMK cascades control cortical axonal and dendritic growth
 (A, B) A scattered plot (orthogonal plot) of datapoints (A) and averages (B) for both axonal and dendritic lengths obtained of individual neurons. Black circles, wildtype (WT). Blue triangles, I γ /CL3 knockout (I γ /CL3 KO). Red squares, CaMKK α / β -double knockout (DKO). Number of neurons: WT, n = 52; I γ /CL3 KO, n = 44; DKO, n = 52. ^{a)} Axon, p<0.001; Dendrite, p<0.001. ^{b)} Dendrite, p<0.01 (one-way ANOVA with Tukey's test comparison with WT). (C, D) Cumulative probability analysis for total axonal length (C) and total dendritic length (D) in neurons from WT, DKO, and I γ /CL3 KO mice. Number of neurons: WT, n = 52; DKO, n = 52; I γ /CL3 KO, n = 44. **, p < 0.01; ***, p<0.001, Kolmogorov-Smirnov test comparison with WT.

(E, F) Cortical neurons (2 days in vitro) from CaMKK α / β -double knockout (DKO), mice (F) showed impaired growth of axons (arrowheads) and dendrites (arrows) as compared with neurons from wildtype (WT) mice (E). Bar, 25 μ m.

(G) Treatment with KN-93, a general CaMK inhibitor, and STO-609, a blocker of CaMKK α / β , the upstream kinases of all CaMKI/IV, from 6 h to 48 h after plating impaired both axonal (arrowheads) and dendritic (arrows) growth. Scale bar, 50 μ m. (H) An orthogonal plot shows a quantitative analysis of axonal and dendritic morphometric parameters from each neuron. Number of neurons: DMSO, n = 48; KN-93, n = 43; STO-609, n = 48. ^{a), b)} Axon, p<0.001; Dendrite, p<0.001 (one-way ANOVA with Tukey's test comparison with DMSO).

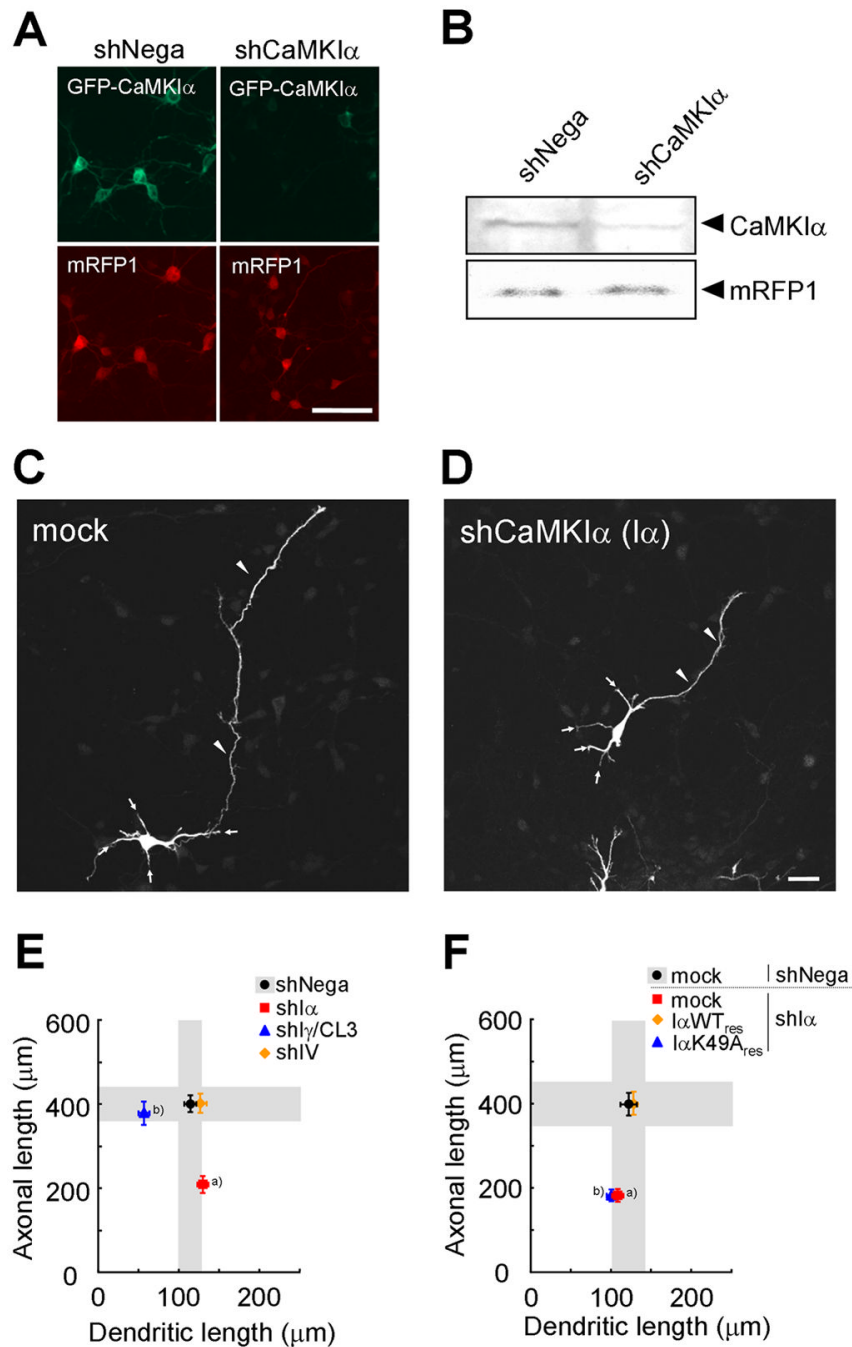


Figure 2. Knockdown of CaMKI α specifically impairs axonal but not dendritic growth
 (A) Efficient downregulation of exogenous GFP-CaMKI α was achieved by a CaMKI α -targeted shRNA vector (shCaMKI α), but not by a control vector (shNega), in rat cortical neurons. The mRFP1 expression, which was driven by a dual promoter in a pSUPER+mRFP1 vector, remained unchanged. Bar, 50 μ m.

(B) Knockdown of endogenous CaMKI α was evaluated by Western blot analysis using an anti-CaMKI α antibody. Rat cortical neurons were transfected with pSUPER-shNega or pSUPER-shCaMKI α by electroporation and the cells were lysed at 2 DIV. shCaMKI α suppressed endogenous CaMKI α while the control mRFP1 expression level remained unchanged.

(C, D) shCaMKI α -expressing rat cortical neurons (shCaMKI α) (D) showed impaired axonal growth (arrowheads), while the dendritic morphology was spared (arrows) as compared with neurons from shNega-expressing rat cortical neurons (shNega) (C). Bar, 25 μ m.

(E) An orthogonal plot of averaged data; n = 15 for all groups. ^{a)} Axon, p<0.001. ^{b)} Dendrite, p<0.001 (one-way ANOVA with Tukey's test comparison with shNega). Bar, 25 μ m.

(F) Introduction of shCaMKI α -resistant wild-type GFP-CaMKI α WT_{res}) successfully rescued the axonal defect elicited by shCaMKI α . In contrast, an shCaMKI α -resistant kinase-inactive GFP-CaMKI α (a K49A_{res} point mutant) was unable to rescue the shCaMKI α phenotype. n = 15 for all groups. ^{a), b)} Axon, p<0.001 (one-way ANOVA with Tukey's test comparison with shNega + mock).

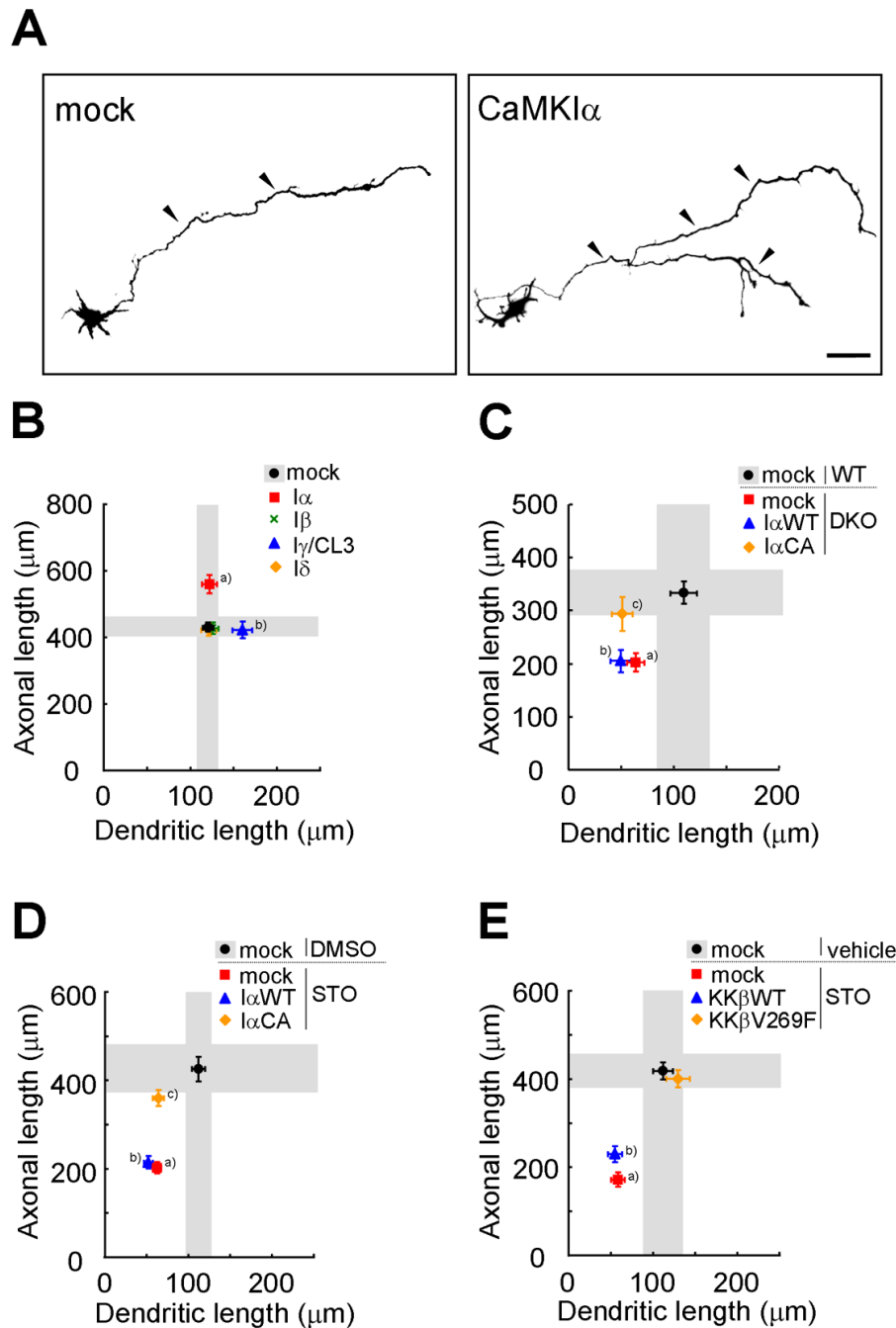


Figure 3. A specific role for a CaMKK-CaMKI α cascade in promoting axonal growth in cortical neurons

(A) Representative images of rat cortical neurons transfected with GFP-CaMKI α . Bar, 50 μ m.

(B) Overexpression of CaMKI α and CaMKI γ facilitated axonal and dendritic growth, respectively. $n = 15$ for all groups. ^{a)} Axon, $p < 0.001$, ^{b)} Dendrite, $p < 0.05$ (one-way ANOVA with Tukey's test comparison with mock).

(C) The axonal growth defect in DKO mice was selectively rescued by co-expression of a constitutively active CaMKI α (CaMKI α CA), but not by a wild-type CaMKI α (CaMKI α WT); the dendritic growth defect was left unaltered. $n = 15$ for all groups. ^{a)} Axon, $p < 0.01$; Dendrite,

p<0.05, ^{b)} Axon, p<0.01; Dendrite, p< 0.001, ^{c)} Dendrite, p<0.01 (one-way ANOVA with Tukey's test comparison with WT + mock).

(D) Only the axonal growth defects due to STO-609 treatment were rescued by expression of a constitutively active CaMKI α (CaMKI α CA), but not of a wild-type CaMKI α CaMKI α WT). Dendritic growth defects remained unchanged. n = 15 for all groups. a), b) Axon, p<0.001; Dendrite, p<0.001, c) Dendrite, p<0.001 (one-way ANOVA with Tukey's test comparison with DMSO + mock).

(E) Both axonal and dendritic growth defects due to STO-609 treatment were rescued by introducing an STO-609-resistant CaMKK β mutant (V269F), but not of a CaMKK β -WT. n = 15 for all groups. a), b) Axon, p<0.001; Dendrite, p<0.01 (one-way ANOVA with Tukey's test comparison with vehicle + mock).

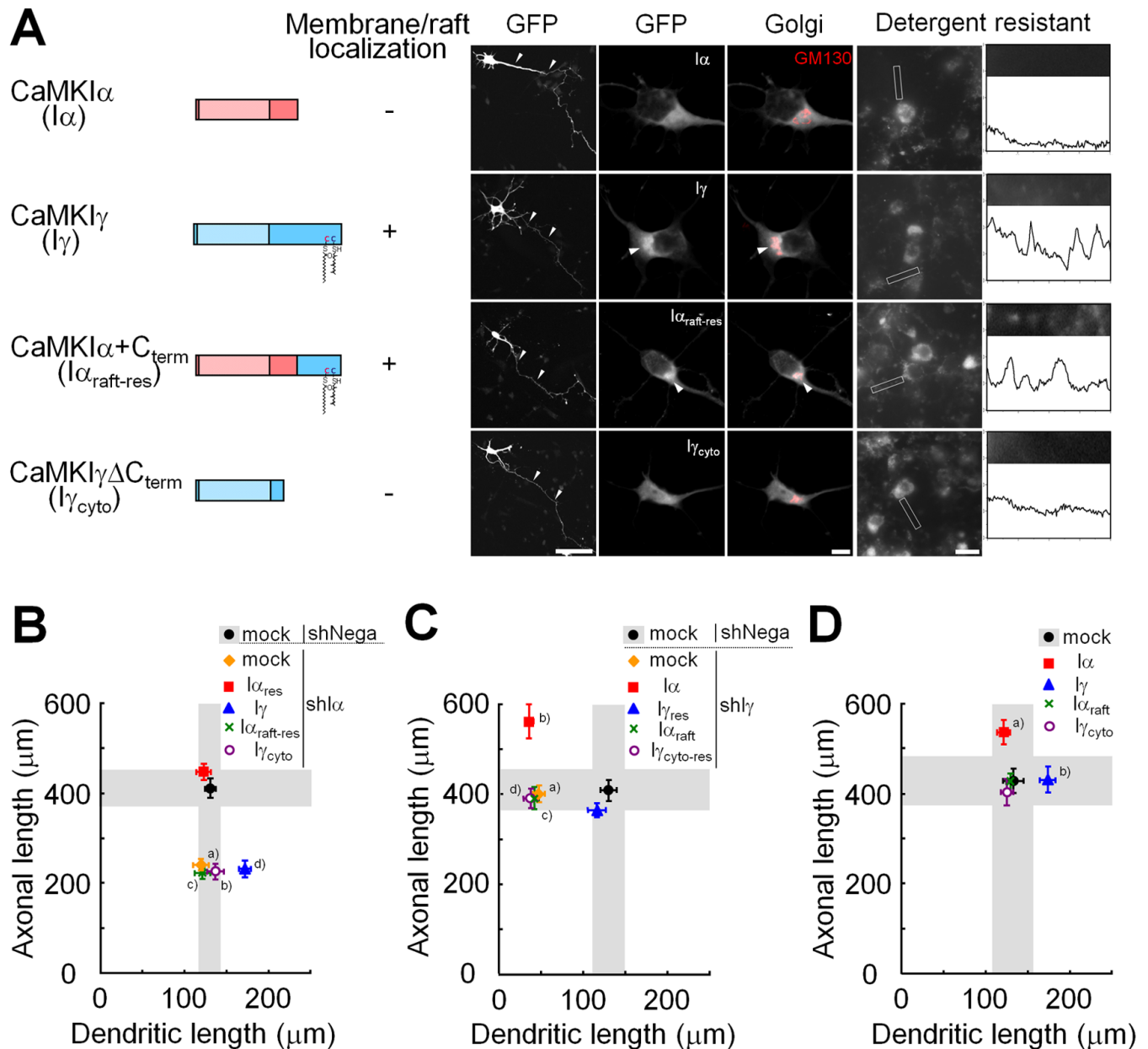


Figure 4. Functional segregation of CaMKK-CaMKIα and CaMKK-CaMKIγ cascades
 (A) The domain structures and subcellular localizations of CaMKIα (Iα), CaMKIγ/CL3 (Iγ) and their chimeras. GFP- CaMKIα (Iα), CaMKIγ/CL3 (Iγ) and their chimeras distribution detected by anti-GFP immunostaining showed co-localization with a Golgi marker, GM130. GFP- Iγ and Iα_{raft-res} signals were also enriched within Golgi (arrowheads). Single representative confocal sections are shown for Golgi localization. GFP-Iγ and -Iα_{raft-res} fluorescence was retained after detergent treatment in a punctate manner in 2 DIV cortical neurons along the dendrites, demonstrating a sizable portion of detergent-resistant GFP-Iγ and Iα_{raft-res} in the dendritic rafts. Line scans of pixel fluorescence, carried out within a chosen field of a 15 μm dendritic segments. Bar, 50 μm (right), 5 μm (middle), 100 (left) μm.

(B) Neither $I\gamma$, $I\alpha_{\text{raft-res}}$, nor $I\gamma_{\text{cyto}}$ were able to rescue the axonal phenotype due to knockdown of CaMKI α . $n = 15$ for all groups. ^{a), b), c)} Axon, $p < 0.001$; ^{d)} Axon, $p < 0.001$, Dendrite, $p < 0.01$ (one-way ANOVA with Tukey's test comparison with shNega + mock).

(C) Neither $I\alpha$, $I\alpha_{\text{raft}}$ nor $I\gamma_{\text{cyto-res}}$ were able to rescue the dendritic phenotype due to knockdown of CaMKI γ /CL3. $n = 15$ for all groups. ^{a), c), d)} Dendrite, $p < 0.001$; ^{b)} Axon, $p < 0.001$, Dendrite, $p < 0.001$ (one-way ANOVA with Tukey's test comparison with shNega + mock).

(D) Overexpression of CaMKI α specifically increased axon length in cortical neurons. $n = 15$ for all groups. $n = 15$ for all groups. ^{a)} Axon, $p < 0.05$; ^{b)} Dendrite, $p < 0.001$ (one-way ANOVA with Tukey's test comparison with mock).

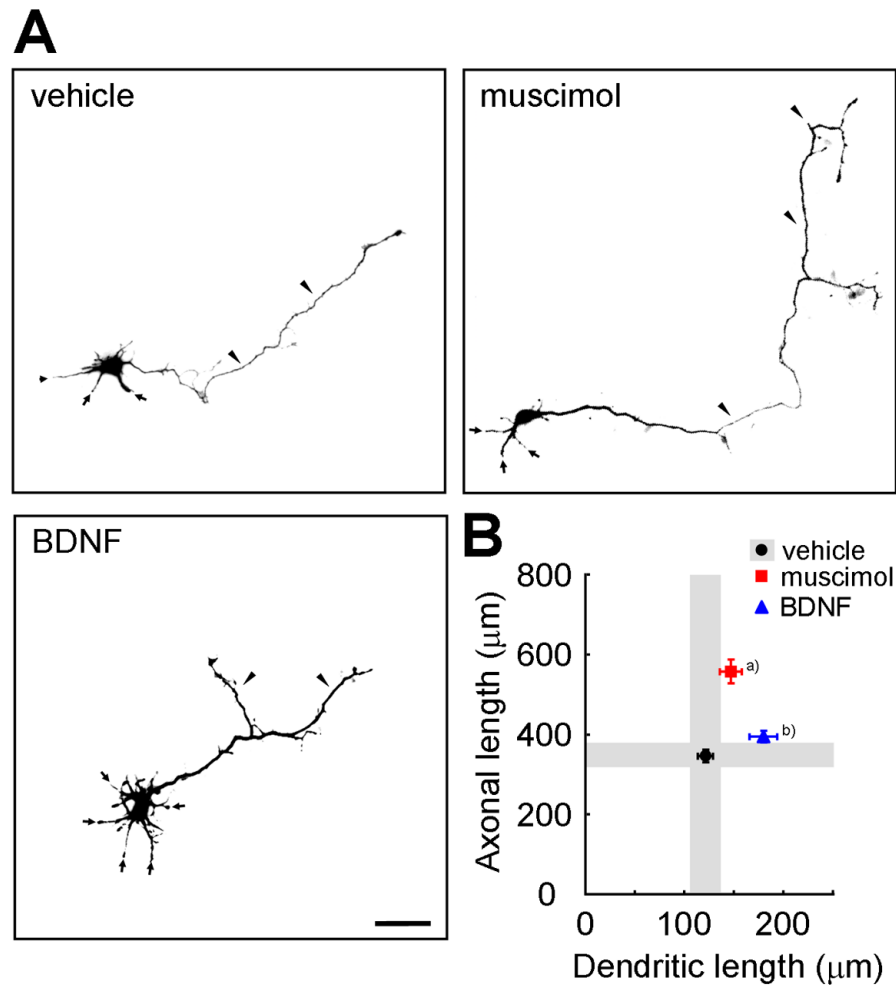


Figure 5. Muscimol, a GABA_A receptor agonist, specifically stimulates elongation of axons in cultured cortical neurons

(A, B) Representative images (A) and ensemble data (B) of immature cortical neurons treated with either muscimol (a GABA_A receptor agonist) or BDNF. Muscimol significantly promoted axonal growth (arrowheads). In contrast, BDNF had no effect on axons, but mainly affected dendrites. Scale bar, 50 μm. n = 15 for all groups. ^{a)} Axon, p<0.001, ^{b)} Dendrite, p<0.01 (one-way ANOVA with Tukey's test comparison with vehicle).

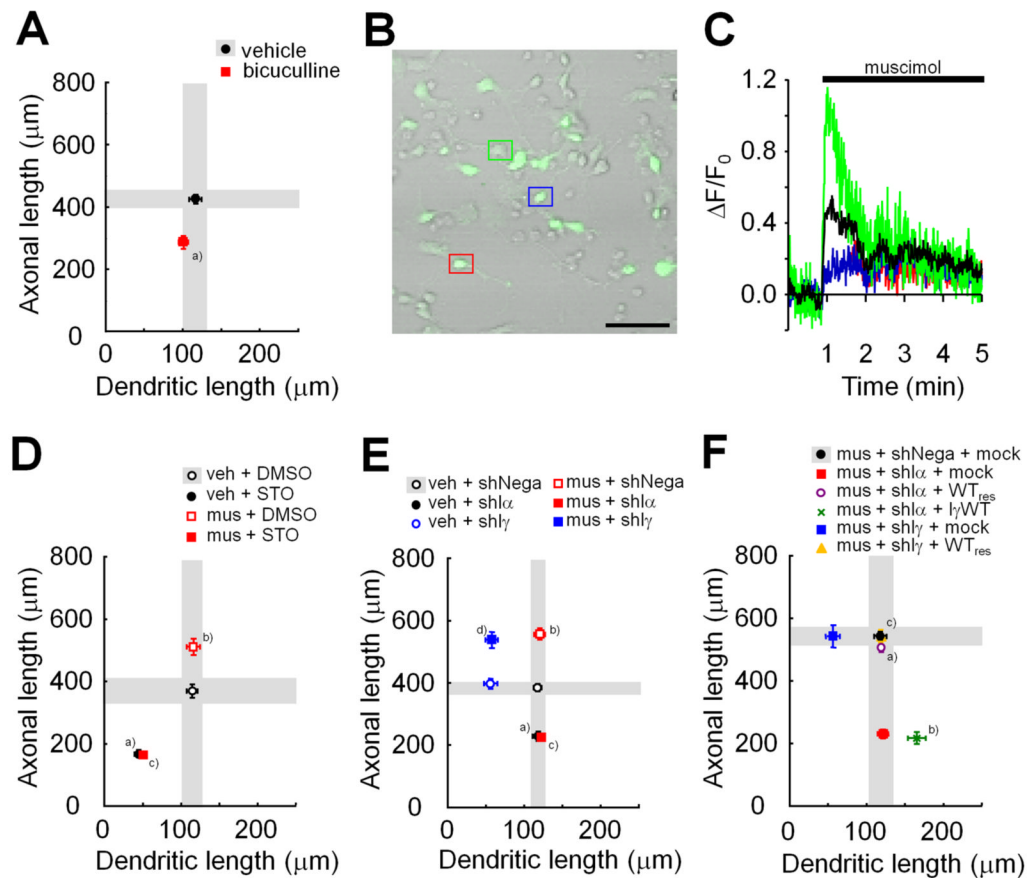


Figure 6. Activation of GABA_A receptors promotes axonal growth via the CaMKK-CaMKII α pathway in immature cortical neurons

(A) Bicuculline, a GABA_A receptor antagonist, blocked axonal growth. $n = 15$ for all groups. ^{a)} Axon, $p < 0.001$; (Student t-test comparison with vehicle).

(B) Embryonic cortical neurons (1 DIV) were loaded with a calcium indicator, Fluo-4AM, and calcium responses were measured by time-lapse imaging. A green fluorescence image was overlaid on a DIC image. Colored boxes indicate the location of cells shown in (C). Scale bar, 50 μm .

(C) Representative calcium responses in individual cells after muscimol administration. Three different types of calcium responses were revealed (green, blue, and red). An averaged response from 10 cells in a microscopic field is revealed in black.

(D) Both basal and muscimol-stimulated axonal growths were suppressed with STO-609, a specific blocker of CaMKK α/β . $n = 15$ for all groups. (Axon: two-way ANOVA, muscimol effect, $F_{1,56} = 14.38$, $p = 0.0004$; drug effect, $F_{1,56} = 225.63$, $p < 0.0001$; muscimol \times drug, $F_{1,56} = 15.79$, $p = 0.0002$). (Dendrite: two-way ANOVA, muscimol effect, $F_{1,56} = 0.39$, $p = 0.5336$; drug effect, $F_{1,56} = 105.76$, $p < 0.0001$; muscimol \times drug, $F_{1,56} = 0.13$, $p = 0.7199$). ^{a), b)} Axon, $p < 0.001$ (comparison with vehicle + DMSO), ^{c)} Axon, $p < 0.001$ (comparison with muscimol + DMSO), n.s. (comparison with vehicle + STO).

(E) CaMKII α knockdown quantitatively inhibited axonal growth induced by muscimol treatment, to an extent similar to that obtained with STO-609. $n = 15$ for all groups. (Axon: two-way ANOVA, muscimol effect, $F_{1,84} = 66.44$, $p < 0.0001$; RNAi effect, $F_{2,84} = 168.04$, $p < 0.0001$; muscimol \times RNAi, $F_{2,84} = 19.96$, $p < 0.0001$). (Dendrite: two-way ANOVA, muscimol effect, $F_{1,84} = 0.23$, $p = 0.6305$; RNAi effect, $F_{2,84} = 61.58$, $p < 0.0001$; muscimol \times RNAi, $F_{2,84} = 0.01$, $p = 0.9888$). ^{a), b)} Axon, $p < 0.001$ (comparison with vehicle +

shNega), ^{c)} Axon, $p < 0.001$ (comparison with muscimol + shNega), n.s. (comparison with vehicle + shI α), ^{d)} Axon, $p < 0.001$ (comparison with vehicle + shI γ), n.s. (comparison with muscimol + shNega).

(F) Introduction of shCaMKI α -resistant wild-type GFP-CaMKI α (WT_{res}) specifically rescued the suppression of muscimol-induced axonal growth triggered by knockdown of CaMKI α . $n = 15$ for all groups. ^{a)} Axon, $p < 0.001$, Dendrite, n.s.; ^{b)} Axon, n.s., Dendrite, $p < 0.001$ (one-way ANOVA with Tukey's test comparison with muscimol + shI α + mock). ^{c)} Axon, n.s., Dendrite, $p < 0.001$ (t test comparison with muscimol + shI γ + mock).

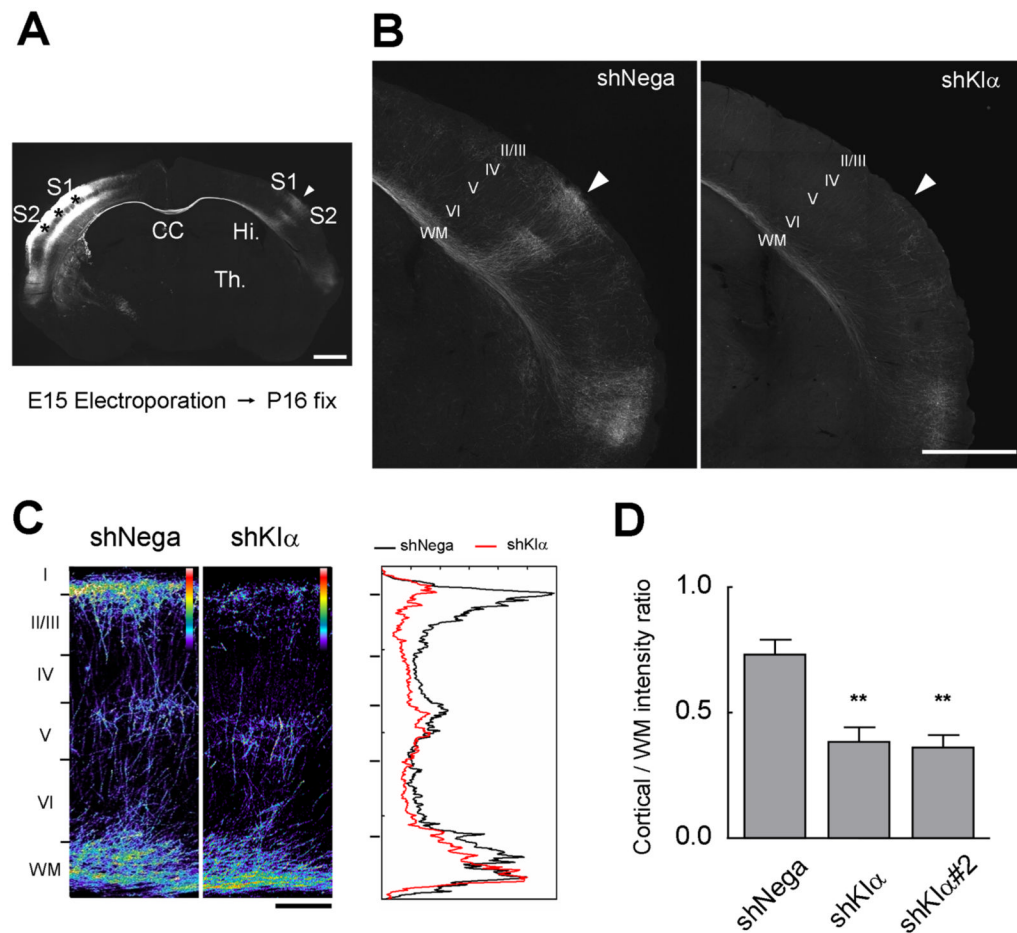


Figure 7. Knockdown of CaMKI α impairs terminal extension of callosal axons *in vivo*

(A) A control coronal section was obtained near the posterior end of the corpus callosum, from a P16 pup electroporated *in utero* with pSUPER-shNega and pCAG-EGFP on E15.5. The somatodendritic regions of layer II/III neurons were strongly labeled (asterisks) in the somatosensory cortex, from which callosal axons projected towards the contralateral cortical areas at the S1/S2 border region (arrowhead). Bar, 1 mm. CC, corpus callosum; Hi, Hippocampus; Th, Thalamus.

(B) Terminal extension of callosal axons into the contralateral cortical layers was severely disrupted in CaMKI α -knockdown neurons (shKI α), although axons were able to reach the white matter (WM) beneath S1/S2 area (arrowhead). Bar, 1 mm.

(C) Axonal extension and terminal branch arborisation were strongly impaired in layers II/III in CaMKI α -knockdown neurons, as illustrated by the magnified images of GFP marker showing the total axonal volumes present in the cortical layers (in pseudocolor), or by a one-dimensional fluorescence intensity profile analysis. Bar: 200 μ m.

(D) Quantification of the cortical wiring defect due to an aberrant terminal axon extension in the cortex. Two independent RNAi constructs (shKI α and shKI α #2) gave similar results.

** $p < 0.01$ (one-way ANOVA with Tukey's test comparison with shNega).